PHOTOREACTIVATION OF ULTRAVIOLET LIGHT INJURY IN GAMETES OF THE SEA URCHIN STRONGYLOCENTROTUS PURPURATUS [1]

PATRICK H. WELLS AND ARTHUR C. GIESE

Department of Biological Sciences, Stanford University, California

The finding that visible light can reverse the deleterious effects of ultraviolet radiations (Kelner, 1949a; Prat, 1936; Whitaker, 1942) has resulted in numerous studies attempting to determine how widespread the phenomenon is and the possible mechanism of action of the ultraviolet and the visible light. The majority of these studies have been concerned with reactivation of ultraviolet killed organisms. Photoreactivation of the killing effect of ultraviolet light has been demonstrated on the bacterium, *Escherichia coli* (Kelner, 1949b; Novick and Szilard, 1949) and on the fungi, *Streptomyces griseus* (Kelner, 1949a), *Penicillium notatum*, *Saccharomyces cerevisiae* (Kelner, 1949b), *Ustilago maydis* (Snyder, unpublished), and others. The phenomenon appears to be basically similar in all these organisms. Exposure of ultraviolet inactivated cells to visible light results in a partial recovery of the lost viability.

There are also reports on the photoreactivation of injurious effects other than killing. Reactivation of ultraviolet induced delay in rhizoid formation in zygotes of the alga *Fucus furcatus* (Whitaker, 1942), photoreactivation of ultraviolet retarded adaptive galactozymase formation in *Saccharomyces cerevisiae* (Swenson and Giese, 1950), and photoreactivation of mutations in *E. coli* to bacteriophage resistance (Novick and Szilard, 1949; Kelner, 1949b) serve as examples. Here too, exposure to visible light after or during ultraviolet irradiation causes partial recovery from the ultraviolet injury.

The experimental conditions necessary to demonstrate photoreactivation in ultraviolet inactivated bacteriophage differ somewhat from the above. The inactivated phage particles alone can not be photoreactivated by visible light, but must be adsorbed onto bacteria before the phenomenon will occur (Dulbecco, 1950).

Blum and his co-workers reported photoreactivation in eggs of the sea urchin *Arbacia punctulata* (Blum, Robinson and Loos, 1949; Blum, Loos, Price and Robinson, 1949). Marshak studied photoreactivation in gametes and zygotes of this sea urchin (Marshak, 1949). He finds no photoreactivation of ultraviolet injury to either gamete alone, but that this does occur if the visible light follows fertilization. He analogizes this situation to that in bacteriophage.

The present study is concerned with photoreactivation in gametes and zygotes of the sea urchin, *Strongylocentrotus purpuratus*. In the literature on photoreactivation, work with monochromatic ultraviolet light is strikingly absent, yet the effects of various wavelengths may be quite different. Therefore, visible light reversal of injury caused to eggs by different monochromatic wavelengths of ultraviolet light

[1] Supported in part by grants from the Rockefeller Foundation.

has been attempted and the region of effective visible light defined. Effects of different dosages of ultraviolet radiations have also been studied. Photoreactivation has been attempted before and after fertilization of the injured gametes, and the effects of visible light on sperm investigated.

Materials and Methods

Sea urchins were collected at Moss Beach, California and later in the breeding season at the mouth of Malpaso Creek near Point Lobos, California, and brought to Stanford University where they were kept at 5° C. for two or three days during which time the gametes were used for experiments. The animals from the Malpaso Creek collecting station, which show a delayed breeding season, were often kept for several days in the aquaria of the Hopkins Marine Station at Pacific Grove, California before transport to Stanford University. There was little deterioration of gametes under these conditions of storage, and no detectable difference between material from the two collecting stations.

Eggs were obtained by cutting open a sea urchin to expose the gonads, rinsing with sea water, and taking some of the eggs which spew out when an ovary is slightly injured. Care was taken to avoid contact of the eggs with body fluids or any organs of the animal. The entire testes were cut out of the males and sperms were taken from these when required. Sperms were used in dilution of 1/400, or else insemination was accomplished by simply touching the tip of a dissecting needle to the testis, and then running this through the egg dish. Only gametes which showed almost 100 per cent fertilization and good early development were used for experiments. Sea water was obtained at the same places as were the urchins. It was filtered immediately and pH determinations showed that it did not change much on standing thereafter.

Dishes were made from the bottoms of new glass vials and were carefully tested to show that they supported normal development before being used in experiments. All operations except the irradiations with the monochromator were performed in a 13° C. constant temperature room. Monochromator irradiations were done at room temperature. Tests showed that such brief exposures to room temperature before fertilization had no detectable effect on the gametes.

The ultraviolet irradiation technique was the same as previously described (Giese, 1938a, b) and the eggs were handled in essentially the same manner. In essence, the source of radiations was a quartz mercury arc used with a natural quartz monochromator. The intensity of a desired wavelength of light was measured at the time of the experiment with a thermopile calibrated against a Bureau of Standards Standard Lamp.

After ultraviolet irradiation, the gametes were carefully screened from visible light, except for controlled exposure during photoreactivation. For this the brilliant light of a 100 Watt G. E. projection spotlamp was used. The lamp was 70 cm. from the eggs and the light passed through 20 cm. of water to remove heat rays and a Corning No. 3060 glass filter to remove short ultraviolet. This filter has a transmission from about λ3700Å through the visible (50 per cent transmission at λ4100Å), and allows good photoreactivation. Blue light from the monochromator (λ4350Å) was used in work with sperm.

Delay in cleavage of the zygotes (formed from union of ultraviolet irradiated gametes of one sex with normal gametes of the opposite sex) was taken as the criterion of injury, and reduction of this ultraviolet induced delay by visible light as photoreactivation. The time at which 50 per cent of an experimental sample of zygotes were divided was taken as the point of cleavage. The method routinely used for obtaining data on the per cent cleavage in a sample was to photograph it at intervals during the course of the experiment in essentially the same way as was done by Blum and Price (1950a, b). This was done with a Leica camera through a Microibso attachment, using an ordinary Leitz monocular microscope with a Leitz No. 2 objective. This gives a field large enough to include one or two hun-

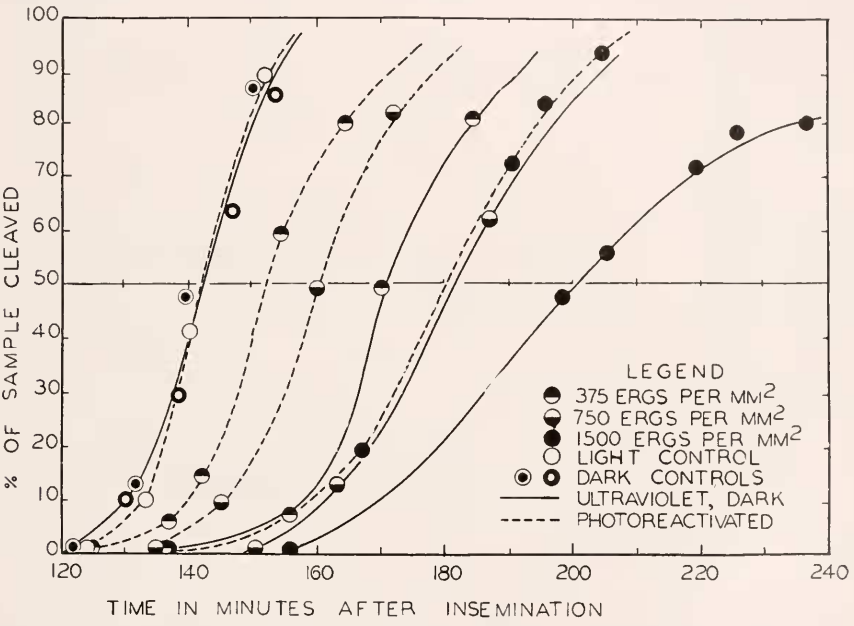

FIGURE 1. Delay in cleavage of sea urchin eggs injured with various dosages of ultraviolet wavelength 2450Å, with and without photoreactivation. All irradiations completed before fertilization. Visible light exposures are the same in each case. Dosage series at λ2537Å and λ2654Å show similar cleavage characteristics.

dred eggs in each photograph. The microscope light was filtered through a Corning No. 3480 Glass filter which transmits only to λ5500Å in the visible (50 per cent transmission at λ5700Å). The filter effectively eliminated photoreactivation from this source and a 5 cm. water cell removed any heat. Exposures of two and one-half seconds on Kodak Microfile film were adequate under the lighting conditions used. The negatives obtained in this way were projected with an enlarger and counts made from them. This method gave a permanent record which could be read at leisure. For critical experiments, the counts were made a number of times and their results were found to be quite repeatable. Experiments were usually repeated three times, and dark and light controls were run on each experiment.

Experimental

In the first group of experiments, the effects of series of dosages of ultraviolet light at λ2450Å, λ2537Å, and λ2654Å were determined. Eggs were irradiated with the indicated dosages of ultraviolet, then exposed to white light from the G. E. spot-lamp for one-half hour, and fertilized with normal sperm. Each series was done with eggs from a single female and sperm from a single male. Data on cleavage were determined as described above, plotted, and the points of 50 per cent cleavage determined. Figure 1 shows the cleavage characteristics of such a series at λ2450Å. The points of 50 per cent cleavage from Figure 1 and from similar series of dosages at λ2537Å and λ2654Å are plotted in Figure 2. In each of these figures, solid lines

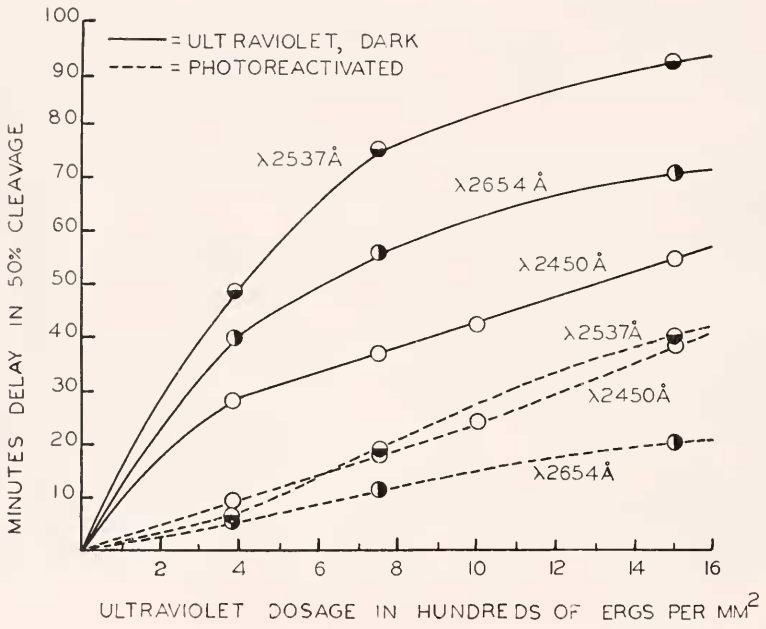

Figure 2. Photoreactivation of injury to eggs caused by wavelengths 2450Å, 2537Å, and 2654Å. Times of 50 per cent cleavage of the samples of eggs are compared here. Visible light dosages were the same in each experiment. All irradiations were completed before insemination.

represent dark controls and ultraviolet injured eggs. The dotted lines represent visible light controls and photoreactivated samples.

Complete dosage series were not run on other wavelengths, but representative dosages were tested. In Table I some of these data are compared, data from Figure 2 being included. Visible light exposure was held constant for all of these experiments.

Photoreactivation occurred for all wavelengths tested and for each trial at any wavelength. Comparison of efficiencies of the process at different wavelengths is difficult because the wavelengths of ultraviolet light vary in the amount of injury caused by a given exposure. The action spectra for ultraviolet light injury to sea urchin gametes have been previously described (Giese, 1946). There is also varia-

TABLE I

Representative photoreactivation data from various wavelengths of ultraviolet light, keeping the visible light dosage constant. Controls are taken as zero delay.

Wavelength of ultraviolet, in Angstrom units	Dosage of ultraviolet, in ergs/mm.²	Cleavage delay, in minutes; ultraviolet	Cleavage delay, in minutes; photoreactivated	Per cent photoreactivation
Control	0	0	0	0
2450	750	37	18	51
2450	1500	55	18	31
2537	750	76	19	75
2537	750	36	6	83
2537	1500	93	40	57
2654	750	56	11	80.5
2654	750	51	14	72.5
2654	1500	71	20	71.7
2804	750	90	26	71
3025	750	37	11	70
3130	6000	14	2	86

tion in the sensitivity to ultraviolet injury in eggs from different females. It seems, however, from the data available (Figure 2 and Table I), that there is strikingly less reactivation from λ2450Å injury than from injury by other wavelengths. Furthermore, there is a marked unilateral effect in raising of the membrane at

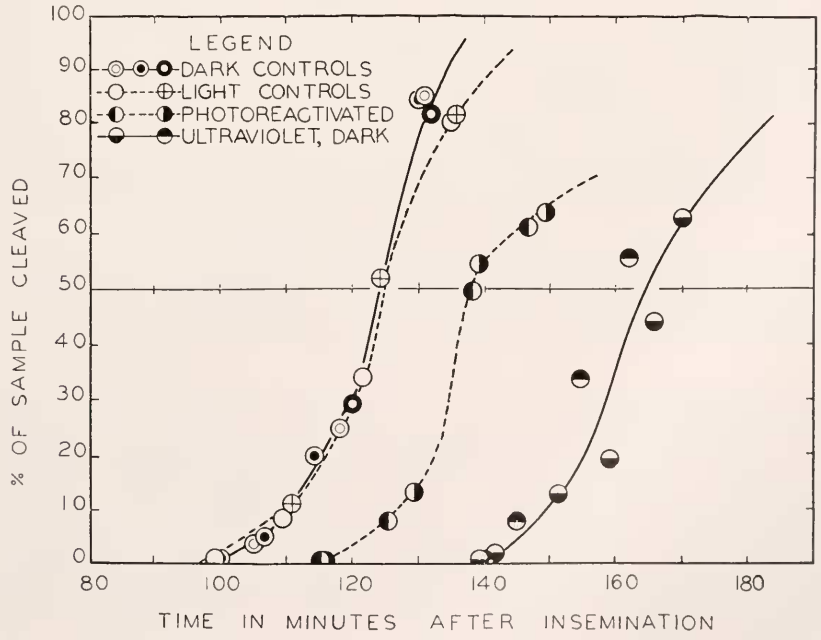

FIGURE 3. Photoreactivation of zygotes formed from eggs injured by 750 ergs/mm.² of λ2654Å ultraviolet light. Visible light exposure of ⅓, used in the experiments of Figure 1 and Figure 2, was immediately after fertilization. Photoreactivation of 65.5 per cent was obtained.

λ2450Å which is not usually apparent at other wavelengths. This suggests that injury from superficially absorbed ultraviolet light is not so readily photoreactivated as injury caused by wavelengths which are more strongly absorbed by nucleoproteins. More data are necessary for a complete analysis of this situation.

In all of the experiments discussed above, eggs were photoreactivated with one-half hour exposures under the G. E. spotlamp, then fertilized with normal sperm. This was found by experimentation to be the minimal dosage which would give an effect reasonably near maximal. If a Corning No. 3389 glass filter was substituted for the Corning No. 3060 filter routinely used on the reactivating lamp, photoreactivation was greatly reduced. The Corning No. 3389 filter transmits only wavelengths longer than λ4100Å (50 per cent transmission at λ4300Å). It has been

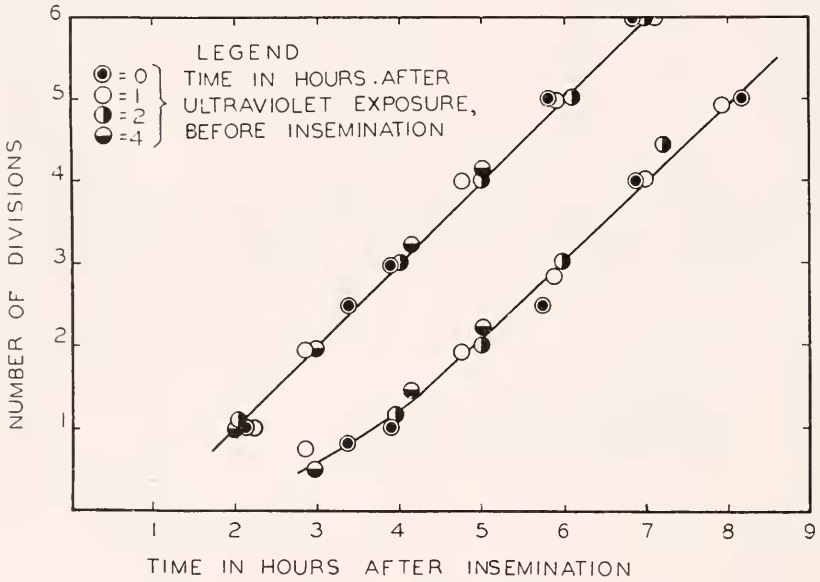

FIGURE 4. Comparison of cleavage rates of sea urchin eggs injured by ultraviolet light, then kept in the dark for various periods of time before insemination. Two experiments are shown.

shown on other organisms that the spectral region λ3660Å to λ4300Å is usually most effective in photoreactivation (Dulbecco, 1950; Kelner, 1950). Our experiments show that in Strongylocentrotus, wavelengths shorter than λ4300Å are most effective.

That fertilization prior to photoreactivation is not required is indicated by all of the experiments thus far described. It was found, however, that after fertilization of injured gametes, photoreactivation of the resulting zygotes was somewhat more easily accomplished. Visible light dosages of ten minutes sufficed to cause reactivation of the same magnitude as was caused by thirty minutes exposure on the unfertilized eggs. Figure 3 shows the results of one experiment on reactivation of zygotes.

Eggs of Strongylocentrotus do not show any measurable increase or decrease in amount of delay caused by an ultraviolet exposure, when they are kept in the dark

in sea water. This was experimentally shown by irradiating samples of eggs with ultraviolet light from a sterilamp (arbitrary exposure of sixteen seconds), and fertilizing portions of the samples at intervals after the irradiation. Samples were followed through the sixth division in order to detect any change in rate of cleavage caused by the delay in fertilization. Portions of each sample were inseminated immediately after irradiation, and at one, two, and four hours thereafter. Figure 4 shows the results of two such experiments. Note that the delay of cleavage for all sample portions of irradiated eggs is practically identical.

Sea urchin sperm are much more sensitive to ultraviolet light injury than the eggs. Fifty ergs of λ2654Å suffices to cause a delay in cleavage of 80 to 100 minutes

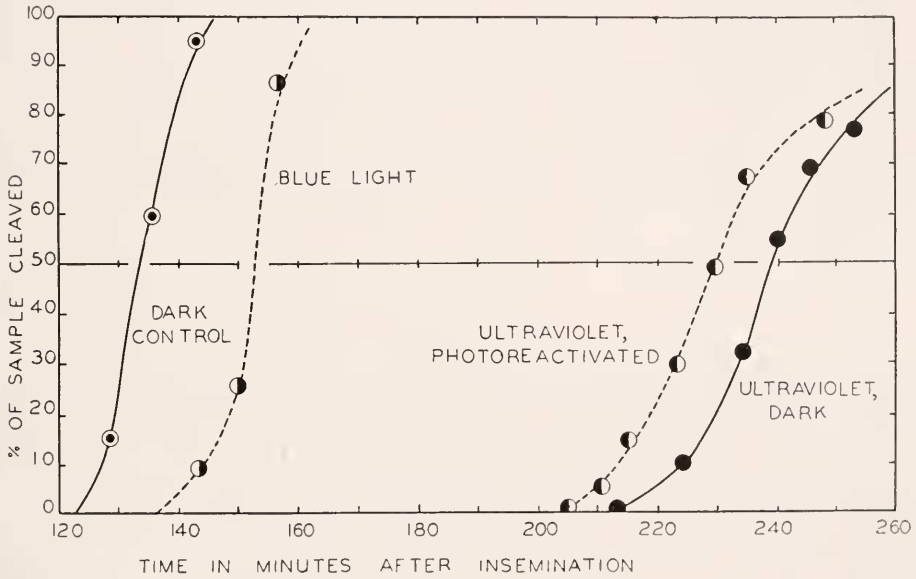

FIGURE 5. Photoreactivation of ultraviolet injured sperm. Sperm were injured with 50 ergs/mm.^{2} of λ2654Å ultraviolet, photoreactivated with 4086 ergs/mm.^{2} of λ4350Å blue light, then used to fertilize normal eggs. Cleavage delay in the resulting zygotes is shown. Note the retardation of cleavage in the blue light control.

in zygotes from such injured sperm. An action spectrum has been worked out which shows that this wavelength is near the peak of efficiency for ultraviolet injury to sperm, and a detailed comparison of ultraviolet effects on eggs and sperm has previously been made (Giese, 1946, 1949). The most obvious effect of ultraviolet light injury to sperm is retardation in the cleavage of zygotes resulting when injured sperm are used to fertilize eggs. Even after quite large dosages of ultraviolet, the sperm remain motile and activate the eggs.

Visible light is also very harmful to sperm. Its effects will be reported in detail elsewhere, but it is necessary to present some of the data here, in connection with photoreactivation in sperm. The visible light injury is qualitatively different from ultraviolet light injury. Sperm exposed to visible light become immotile and fail

to activate the eggs. Sensitivity varies with gametes from different individuals. Usually the dosage which will kill nearly 100 per cent of the sperm is between 20,000 ergs/mm.^{2} and 40,000 ergs/mm.^{2} for monochromatic blue light of λ4350Å (the wavelength used in photoreactivation experiments with sperm). Sublethal dosages cause a delay in cleavage of the zygotes formed from sperm so injured. This delay is of a lower order of magnitude, however, than the ultraviolet induced delay.

The injury caused by ultraviolet light on sperm appears to be different from the injury caused by visible light. Visible light of low intensity might, therefore, cause photoreactivation of ultraviolet induced delay in cleavage, even though the visible light is itself injurious.

In order to test this, sperm suspensions of 1/400 were irradiated in a quartz vessel in the monochromator with 50 ergs/mm.^{2} of λ2654Å ultraviolet, followed by sublethal dosages of blue λ4350Å (4000 to 5000 ergs/mm.^{2}), appropriate controls being run. Normal eggs were then fertilized with these sperm, and the time of cleavage determined. Photoreactivation was in fact demonstrated. Figure 5 shows one such experiment. This was repeated three times with similar results. Note the retardation of cleavage in the blue light control. In spite of this injury, and in spite of the relatively low dosage of visible light used, a photoreactivation of ten minutes is observed.

Discussion

Most previous studies on photoreactivation have been with a sterilamp as the source of ultraviolet radiations. Such sterilamps give off mixed wavelengths of ultraviolet with maximum intensity at λ2537Å. The data presented here indicate that cleavage delay caused by any of the wavelengths of ultraviolet tried is photoreversable, but that λ2450Å injury is less so than injury by wavelengths longer than this. λ2450Å is strongly absorbed by surface proteins, as indicated by the unilateral effect obtained in raising of the membranes when eggs are injured by this wavelength. Several wavelengths tested are much more selectively absorbed by nucleoproteins. It is suggested, then, that injury to nucleoproteins is most strongly photoreactivated. This should be more adequately investigated. It is of interest to note that wavelengths λ2654Å and longer do not produce appreciable amounts of ozone. Therefore, the reversable injury is not due to ozone formed by the ultraviolet light.

Marshak (1949) has found that in Arbacia the injured gametes cannot be photoreactivated, but that zygotes formed from injured gametes are quite sensitive. The data on Strongylocentrotus do not agree with these findings. Either there is a great difference in these two sea urchins with respect to photoreactivation, or the dissimilarities must be accounted for by differences in technique. Since the injured eggs in Strongylocentrotus are less sensitive than zygotes formed from them, perhaps the visible light illumination periods used on Arbacia eggs were too short to cause measurable photoreactivation. Failure of ultraviolet injured sperm to be photoreactivated may have been due to visible light injury to them.

It has been suggested (Novick and Szilard, 1949) that the injurious effect of ultraviolet light may be due to the formation of some sort of poison. Since photo-

reactivation is never complete and is possible only for a time after ultraviolet irradiation, they suggest that the poison has two forms, one stable to visible light and one photolabile. As time passes after injury, the second is largely transformed to the first. Hertel (1905) discussed the possibility of the ultraviolet effect being due to the formation of a poison. He found that if one of a pair of blastomeres was irradiated, the second would be effected, provided the cleavage plane had not yet entirely separated the two. The second blastomere, he argued, was affected by some toxic substance (poison) produced in the first and diffusing into the second.

If poison production is, in fact, the mechanism by which cells are injured by ultraviolet light, this poison must be of a size or form which prevents diffusion through the cell membrane. Hertel's experiment works only if the cleavage plane does not completely separate the blastomeres. Photolyzed eggs added to normal ones do not in any measurable way affect their cleavage. Ultraviolet light injured eggs of Strongylocentrotus do not show recovery, even after standing for hours in sea water. If the injurious substance were able to diffuse into or out of the cell through the membrane, it should certainly be demonstrable by these experiments.

Synthetic processes of the cell seem to be quite sensitive to ultraviolet radiation injury (Giese, 1950; Swenson and Giese, 1950). It can be hypothesized that the photoreversable effects of these radiations, such as killing, inhibition of adaptive enzyme formation, and cleavage delay, are all manifestations of a basic disruption of the synthetic processes of the cell. Failure to obtain complete photoreactivation suggests a multiple effect of ultraviolet light.

Summary

1. Ultraviolet radiation induced cleavage delay in eggs of the sea urchin *Strongylocentrotus purpuratus* can be reduced by exposure to visible light before fertilization.

2. Photoreactivation by visible light occurs in injury by any of the wavelengths of ultraviolet light which we tested: λ2450Å, λ2537Å, λ2654Å, λ2804Å, λ3025Å, λ3130Å. The phenomenon is less pronounced at λ2450Å than at the other wavelenths tried.

3. Wavelengths shorter than λ4300Å are most effective in photoreactivation. The minimum visible light exposure giving an effect near maximal is determined.

4. Zygotes formed from ultraviolet injured eggs are more readily photoreactivated than the unfertilized eggs.

5. Ultraviolet irradiated eggs show no increase or decrease in injury when kept in the dark for several hours before fertilization.

6. Visible light is injurious to sperm.

7. Sperm injured by ultraviolet radiations can be photoreactivated, even though the visible light is itself harmful.

8. The results are compared with photoreactivation data in the literature and discussed with reference to possible mechanisms of action for the phenomena observed.

We are indebted to Dr. L. R. Blinks and Dr. R. L. Bolin for many courtesies extended to us at the Hopkins Marine Station, and especially to Dr. C. S. Yocum for help in obtaining suitable biological material.

LITERATURE CITED

BLUM, H. F., J. C. ROBINSON AND G. M. LOOS, 1949. Effects of ultraviolet radiation on the rate of cell division in Arbacia eggs, and the enhancement of recovery by visible light. *Anat. Rec.*, **105**: 524.

BLUM, H. F., G. M. LOOS, J. P. PRICE AND J. C. ROBINSON, 1949. Enhancement by "visible" light of recovery from ultraviolet irradiation in animal cells. *Nature*, **164**: 1011.

BLUM, H. F. AND J. P. PRICE, 1950a. Delay of cleavage of the Arbacia egg by ultraviolet radiation. *J. Gen. Physiol.*, **33**: 285–304.

BLUM, H. F. AND J. P. PRICE, 1950b. Time relationships in the cleavage of the normal fertilized egg of *Arbacia punctulata*. *J. Gen. Physiol.*, **33**: 305–310.

DULBECCO, R., 1950. Experiments on photoreactivation of bacteriophages inactivated with ultraviolet radiation. *J. Bact.*, **59**: 329–347.

GIESE, A. C., 1938a. The effects of ultraviolet radiation of λ2537Å upon cleavage of sea urchin eggs. *Biol. Bull.*, **74**: 330–341.

GIESE, A. C., 1938b. The effects of ultraviolet radiations of various wavelengths upon cleavage of sea urchin eggs. *Biol. Bull.*, **75**:238–247.

GIESE, A. C., 1946. Comparative sensitivity of sperm and eggs to ultraviolet radiations. *Biol. Bull.*, **91**: 81–87.

GIESE, A. C., 1949. Conference on problems of general and cellular physiology relating to fertilization. I. Activation of eggs, fertilization and early development as affected by ultraviolet rays. *Amer. Nat.*, **83**: 165–183.

GIESE, A. C., 1950. The action of ultraviolet radiation on protoplasm. *Physiol. Reviews* (in press).

HERTEL, E., 1905. Über die Einwirkung von Lichtstrahlen auf den Zellteilungsprozess. *Zeitschr. fallg. Physiol.*, **5**: 535–565.

KELNER, A., 1949a. Effect of visible light on the recovery of *Streptomyces griseus* conidia from ultraviolet irradiation injury. *Proc. Nat. Acad. Sci.*, **35**: 73–79.

KELNER, A., 1949b. Photoreactivation of ultraviolet-irradiated *Escherichia coli*, with special reference to the dose-reduction principle and to ultraviolet-induced mutation. *J. Bact.*, **58**: 511–522.

KELNER, A., 1950. Action spectra for photoreactivation. *Bact. Proceedings*, 1950: 53.

MARSHAK, A., 1949. Recovery from ultra-violet light-induced delay in cleavage of Arbacia eggs by irradiation with visible light. *Biol. Bull.*, **97**: 315–322.

NOVICK, A. AND L. SZILARD, 1949. Experiments on light-reactivation of ultra-violet inactivated bacteria. *Proc. Nat. Acad. Sci.*, **35**: 591–600.

PRAT, S., 1936. Strahlung und antagonistische Wirkungen. *Protoplasma*, **26**: 113–149.

SNYDER, J. B., personal communication.

SWENSON, P. A. AND A. C. GIESE, 1950. Photoreactivation of galactozymase formation in yeast. *J. Cell. Comp. Physiol.* (in press).

WHITAKER, D. M., 1942. Counteracting the retarding and inhibitory effects of strong ultraviolet on Fucus eggs by white light. *J. Gen. Physiol.*, **25**: 391–397.